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14. ABSTRACT The purpose of this study is to develop a real-time PCR-based high throughput detection and identification method for important wound pathogens. This will enable us to provide accurate identification a great deal sooner than has been possible in the past. This is likely to result in improved clinical outcome, less chance of bacterial resistance, and cost savings. To date, we have developed over 20 group- or species-specific primer/probe sets for pathogens encountered in wound infection. The specificities of the primer/probe sets were successfully evaluated with a battery of bacteria. The evaluation of sensitivity is underway. When the entire set of primers and probes has been completed, we will be able to detect, identify and semi-quantitate the important wound pathogens within 8 hours using this TaqMan PCR assay.				
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INTRODUCTION

Wound infection remains an important problem in both military and civilian life despite availability of a wide array of antimicrobial agents and improvements in surgical technique. Wound infection leads to morbidity and, at times, mortality. Studies have shown that infection of post-surgical wounds adds 10 days to the period of hospitalization and approximately \$4,000 to the cost [1,2]. Severe trauma and foreign bodies add to the difficulties in management of military wounds and some civilian wounds. Other problems in wound management relate to the increasing numbers of immunosuppressed patients, the use of extensive surgical procedures in certain situations, and the increasing incidence of microorganisms resistant to antimicrobial agents.

Most wound infections involve both aerobic or facultative bacteria and anaerobic or microaerophilic bacteria. The latter category of organisms often have fastidious growth requirements and may therefore be readily overlooked. The difficulties and time required for good anaerobic bacteriology, along with decreases in available funding, have led many laboratories to do abbreviated workups for anaerobes or to eliminate anaerobic bacteriology entirely. As few as 50 to 75% of anaerobes from various sources are characterized satisfactorily, according to a recent survey [3] and 27% of hospital microbiology laboratories indicated that they never identified anaerobes. Furthermore, virtually all laboratories depend on phenotypic testing alone for identification, typically using commercial kits. These conventional identification protocols are not only laborious and time-consuming, but also often result in inconclusive or even inaccurate identification. In addition, they are culture-dependent and thus more time-consuming. Anaerobic bacteria grow more slowly, but more importantly it takes considerable time to get the various elements of a mixed infection (commonly involving 4 to 12 bacteria) isolated in pure culture so that identification and antimicrobial susceptibility testing can proceed. These delays and limitations can lead to inappropriate antibiotic use in wound infection patients or use of unnecessarily broad-spectrum agents, which contributes to increasing rates of antibiotic resistance. If empirical treatment is ineffective, the patient may deteriorate. The development and application of molecular diagnostic techniques has initiated a revolution in the diagnosis and monitoring of infectious diseases [4]. Rapid diagnosis can be achieved by the direct detection of characteristic bacterial genes in clinical specimens. In recent years, real-time PCR has been applied successfully in the medical field, for example in the quantitation of various DNA and RNA viruses in patients [5-9], in the detection of gene amplification [10], gene mutations [11,12], or chromosomal rearrangements, in the quantitation of gene expression [10,12], and in quantitation of particular pathogens in clinical specimens [13-19]. Here, we describe the use of real-time PCR (TaqMan) assay for high throughput detection of wound infection pathogens.

BODY

[1]: Key pathogens involved in wound infections:

Anaerobes

Gram-positive cocci

Peptostreptococcus micros, P. asaccharolyticus, P. anaerobius, Finegoldia magna

Gram-negative rods:

Prevotella spp., *P. melaninogenicus*, *P. nigrescens*, *P. bivia*, *P. disiens*

Fusobacterium spp., *F. nucleatum*, *F. mortiferum*

Bacteroides fragilis group: *Bacteroides fragilis*, *B. thetaiotaomicron*, *B. vulgatus*, *B. ovatus*, *B. uniformis*, *B. distasonis*

Bacteroides gracilis

Bilophila wadsworthia

Porphyromonas macacae

Gram-positive rods:

Actinomyces odontolyticus, *A. viscosus*, *A. naeslundii*, *A. israelii*

Clostridium perfringens, *C. ramosum*, *C. sordellii*, *C. novyi*

C. clostridioforme group: *C. bolteae*, *C. clostridioforme*, and *Clostridium hathewayi*

Aerobes

Gram-positive cocci:

Streptococci:

Group A streptococci (*Streptococcus pyogenes*)

Group B streptococci (*Streptococcus agalactiae*)

Streptococcus anginosus group: *S. anginosus*, *S. constellatus* subsp. *constellatus*, *S. intermedius*

Staphylococci:

Staphylococcus spp., *S. aureus*, *S. epidermidis*

MRSA (Methicillin Resistant *Staphylococcus aureus*)

MSSA (Methicillin Sensitive *Staphylococcus aureus*)

PVL toxin (Panton Valentine Leukocidin toxin)

Enterococci:

Enterococcus faecium, *E. faecalis*

Gram-negative rods:

Enterobacter cloacae, *E. aerogenes*

Escherichia coli

Klebsiella oxytoca, *K. pneumoniae*

Pseudomonas aeruginosa

[2] TaqMan probe technology:

The Taqman probe technology combines the features of rapid PCR and real-time detection of an amplification product. It uses the 5'-nuclease assay and FRET (fluorescent resonance energy transfer). The hydrolysis probe is conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact. However, upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolyzed by the *Taq* polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochromes.

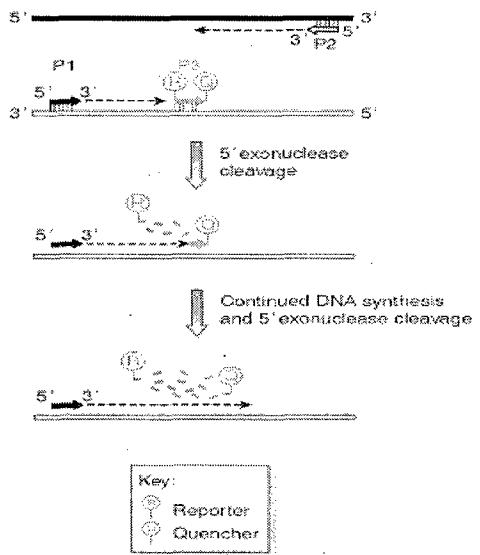


Fig.1. In addition to two conventional PCR primers, P1 and P2, which are specific for the target sequence, a third primer, P3, is designed to bind specifically to a site on the target sequence downstream of the P1 binding site. P3 is labelled with two fluorophores, a reporter dye (R) is attached at the 5' end, and a quencher dye (D), which has a different emission wavelength to the reporter dye, is attached at its 3' end. Because its 3' end is blocked, primer P3 cannot by itself prime any new DNA synthesis. During the PCR reaction, *Taq* DNA polymerase synthesizes a new DNA strand primed by P1 and as the enzyme approaches P3, its 5'–3' exonuclease activity progressively degrades the P3 primer from its 5' end. The end result is that the nascent DNA strand extends beyond the P3 binding site and the reporter and quencher dyes are no longer bound to the same molecule. As the reporter dye is no longer in close proximity to the quencher, the resulting increase in reporter emission intensity is easily detected

Real-time PCR has several advantages over conventional PCR. It focuses on the logarithmic phase of product accumulation rather than on the end-product abundance. Therefore, it is more accurate since it is less affected by amplification efficiency or depletion of a reagent. In addition, it has an increased dynamic range for quantification of target sequence (at least 5 orders of magnitude). Furthermore, without any post-PCR manipulation of the samples, cross-contamination between samples is greatly reduced. Finally, PCR results can be obtained within 2 hours.

(1). ABI PRISM® 7500 Sequence Detection System

ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif.) was purchased, set up and standardized. This system integrates a PCR-based assay with laser scanning technology to excite fluorescent dyes present in the specially designed TaqMan® probes. It is a fully integrated system for real-time detection of PCR. The system includes a built-in thermal cycler, a laser to induce fluorescence, CCD (charge-coupled device) detector, real-time sequence detection software, and TaqMan® reagents for the fluorogenic 5' nuclease assay. All the personnel involved in this project were trained.

(2). TaqMan Primer and Probe design:

Generally, putative genus- or species-specific primer and probe sets were chosen by examining the alignment of the target sequences with sequences of similar organisms

and selecting sequences unique to the organism(s) in question. Sequence alignment was done by using CLUSTAL-W [20] (<http://genome.kribb.re.kr>). Regions that are unique to sequences representing different genera or species were selected for primer and probe design. All primer and probe sets were subjected to the guidelines established by Applied Biosystems by using Primer Express 2.0 (Applied Biosystems, Foster City, Calif.) The primer and probe sequences were analyzed for T_m (melting temperature), secondary structure formation, G+C content, and primer-dimer formation with the NetPrimer analysis software <http://www.premierbiosoft.com/netprimer>). We compared potential candidates for PCR primers and probes to the aligned SSU_rRNA database of the Ribosomal Database Project using the CHECK_PROBE utility [21] and to all available sequences by using the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST) [22]. All the primers and probes designed to date are summarized in **Table 1**.

16S rRNA gene:

The genus- or species-specific primer and probe sets finished to date in this study were designed from the 16S rRNA gene for all the following bacteria:

Bacteroides fragilis group species: *B. fragilis*, *B. stercoris*, *B. vulgatus* and *B. thetaiotaomicron*
Bilophila wadsworthia
Prevotella species including *P. disiens*, *P. bivia*, *P. melaninogenica*, and *P. nigrescens*
Porphyromonas macacae (& *Porphyromonas salivosa*)
Gram-positive anaerobic cocci (GPAC) including *Peptostreptococcus anaerobius*, *P. micros* and *Finegoldia magna*
Actinomyces odontolyticus
Pseudomonas aeruginosa

Spy 1258 gene:

A *S. pyogenes*-specific gene (*Spy 1258*) that encodes a putative transcriptional regulator [23] was used for *Streptococcus pyogenes* (Group A streptococcus) specific-specific primer and probe design. It was noted that a stretch of nucleotides identical to *Spy 1258* (nt. 6651-7193) was also found in the complete genome of *S. pyogenes* M3 strains MGAS315 (GenBank accession No. AE014154) and SSI-1 (GenBak accession No. AP005144) as well as M18 strain MGAS8232 (GenBank accession No. AE010045). However, the *Spy 1258* gene sequence was clearly absent in other bacterial genomes available at GenBank. The primer and probe set was subjected to the guidelines established by Applied Biosystems (Foster City, Calif.) by using Primer Express software (V.2.0).

sip gene:

The primers and probes specific for group B streptococcus (GBS)-*Streptococcus agalactiae* were designed using the *sip* gene [24] which encodes Sip-surface immunogenic protein as this protein is universally expressed in GBS. *S. agalactiae*-

specific primers and probe were designed using Primer Express 2.0 from Applied Biosystems.

pbp2b gene:

The partial penicillin-binding protein 2B gene (*pbp2b* gene) [25] was used for the anginosus group streptococci (AGS)(formerly called “*Streptococcus milleri*”)-specific primer and probe design. The primers and probes were designed according to the alignment analysis of *pbp2b* gene sequences of *Streptococcus anginosus* strain MAS624 (GenBank Accession No. AY289802), *Streptococcus intermedius* strain ATCC 27335 (AY289801), *Streptococcus constellatus* subsp. *pharyngis* strain MM9889a (AY289800), *Streptococcus anginosus* strain ATCC 33397 (AY289798), *Streptococcus constellatus* subsp. *constellatus* strain ATCC 27823 (AY289799) with the homologous genes of *Streptococcus mitis* (Z22182), *Streptococcus pneumoniae* (Z22184), *Streptococcus mutans* (AE014903) and *Streptococcus agalactiae* (AL766847).

tuf gene:

The *tuf* gene encodes the elongation factor Tu, which is an essential constituent of the bacterial genome [26]. The *tuf* gene sequences of *Staphylococcus* species (*S. aureus* [AF298796], *S. epidermidis* [AF298800], *S. saprophyticus* [AF298804], *S. haemolyticus* [AF298801], *S. hominis* [AF298802], *S. lugdunensis* [AF298803], *S. simulans* [AF208805], *S. warneri* [AF298806], *S. xylosus* [AY763438], *S. sciuri* [AY763434], *S. cohnii* subsp. *ureolyticus* [AY298799] and *S. capitis* [AF298798]) that were available from GenBank were analyzed by the software mentioned above.

nuc gene:

S. aureus strains produce an extracellular thermostable nuclease (TNase) with a frequency similar to that at which they produce coagulase. TNase protein has been well characterized and its gene, the *nuc* gene, has been cloned and sequenced [27]. The *nuc* gene is unique to *S. aureus* and it was used for primer and probe design.

(3). TaqMan real-time PCR:

Real-time PCR was performed on the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif.). After primers and probes were designed, they were synthesized by Sigma-genosys. Probes were labeled with the reporter dye 6-carboxyfluorescein (6'-FAM) at the 5' end and with the quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. TaqMan real-time PCR was performed with 3 μ l of DNA, 12.5 μ l of 2X TaqMan PCR master mix (Applied Biosystems, Foster City, Calif.), 900 nmol of each primer, and a 200-nmol probe in a final volume of 25 μ l. Thermal cycling conditions were as follows: 2 min at 50°C, 10min at 95°C followed by 45 repeats of 15 s at 95°C, and 1min at 60°C. Data collection was performed during each annealing phase.

Table 1. Primers and probes included in this study

Target organism	Sequence of primer or probe genes (5' end labeled with FAM-6 and 3' end labeled with TRAMA)	Target	Tm	GC%	Length	Reference
* <i>B. fragilis</i>	Bfrag-F: 5'-GCGGGGTGACCGGTATGCTAAT-3', Bfrag-R: 5'-TCACGAAGTCGGTTGCA-3', Bfrag-P: 5'-CCAAAATCCTCTCTAGGATCGAAG-3', Bthe-F: 5'-CGGGCTTAATTGCAATTG-3', Bthe-R: 5'-CCATGCAGCACCTCACATT-3', Bthe-P: 5'-ATAATCTGGAAACAGGTTAGCGCAAGG-3', Bster-F: 5'-TTGCAACTGACTGAATCGGAA-3', Bster-R: 5'-CTGACCAACCATGCAGCA-3', Bster-P: 5'-CGGTTCTTCCTCGGACAGTTGTGAA-3', Bvul-F: 5'-CGGGCTTAATTCCAGATGA-3', Bvul-R: 5'-CATGCAGCACCTCACAGAT-3', Bvul-P: 5'-GGTGAAGGCCGTAAGCCGCAAGG-3', Bwads-F: 5'-GGCTGGAAACGGTGCCTAA-3', Bwads-R: 5'-GGACTCATCCTTAAGGGATAGC-3', Bwads-P: 5'-CGAATACGCTCCGATTATCATTTGGGG-3', Prev-F: 5'-CCAGCCAAGTAGCGTGCA-3', Prev-R: 5'-TGGACCTCCGTATTACCGC-3', Prev-P: 5'-CAAATCTGATGCCGTCATCGAAGACTATGC-3', Prig-F: 5'-GGCCTAATACCCGATGTGTT-3', Prig-R: 5'-ACGCCATCCCCATCCCTTA-3', Prig-P: 5'-GACGGCATCCGATATGAAACAAAGG-3', Pseu-F: 5'-TGGGATCACCACCTCTACTT-3', Pseu-R: 5'-TTTCGGTGTACGTCAGA-3', Pseu-P: 5'-AGTACGACAGCTCCGACCTGAAGCCG-3',	16s rRNA	60.0 58.4	55.0 55.5	20 18	This study
* <i>B. thetaiotaomicron</i>	Bthe-F: 5'-CCATGCAGCACCTCACATT-3', Bthe-P: 5'-ATAATCTGGAAACAGGTTAGCGCAAGG-3', Bster-F: 5'-TTGCAACTGACTGAATCGGAA-3', Bster-R: 5'-CTGACCAACCATGCAGCA-3', Bster-P: 5'-CGGTTCTTCCTCGGACAGTTGTGAA-3', Bvul-F: 5'-CGGGCTTAATTCCAGATGA-3', Bvul-R: 5'-CATGCAGCACCTCACAGAT-3', Bvul-P: 5'-GGTGAAGGCCGTAAGCCGCAAGG-3', Bwads-F: 5'-GGCTGGAAACGGTGCCTAA-3', Bwads-R: 5'-GGACTCATCCTTAAGGGATAGC-3', Bwads-P: 5'-CGAATACGCTCCGATTATCATTTGGGG-3', Prev-F: 5'-CCAGCCAAGTAGCGTGCA-3', Prev-R: 5'-TGGACCTCCGTATTACCGC-3', Prev-P: 5'-CAAATCTGATGCCGTCATCGAAGACTATGC-3', Prig-F: 5'-GGCCTAATACCCGATGTGTT-3', Prig-R: 5'-ACGCCATCCCCATCCCTTA-3', Prig-P: 5'-GACGGCATCCGATATGAAACAAAGG-3', Pseu-F: 5'-TGGGATCACCACCTCTACTT-3', Pseu-R: 5'-TTTCGGTGTACGTCAGA-3', Pseu-P: 5'-AGTACGACAGCTCCGACCTGAAGCCG-3',	16s rRNA	57.1 59.0	48.2 50.0	29 20	This study
* <i>B. stercoris</i>	Bster-F: 5'-TTGCAACTGACTGAATCGGAA-3', Bster-R: 5'-CTGACCAACCATGCAGCA-3', Bster-P: 5'-CGGTTCTTCCTCGGACAGTTGTGAA-3', Bvul-F: 5'-CGGGCTTAATTCCAGATGA-3', Bvul-R: 5'-CATGCAGCACCTCACAGAT-3', Bvul-P: 5'-GGTGAAGGCCGTAAGCCGCAAGG-3', Bwads-F: 5'-GGCTGGAAACGGTGCCTAA-3', Bwads-R: 5'-GGACTCATCCTTAAGGGATAGC-3', Bwads-P: 5'-CGAATACGCTCCGATTATCATTTGGGG-3', Prev-F: 5'-CCAGCCAAGTAGCGTGCA-3', Prev-R: 5'-TGGACCTCCGTATTACCGC-3', Prev-P: 5'-CAAATCTGATGCCGTCATCGAAGACTATGC-3', Prig-F: 5'-GGCCTAATACCCGATGTGTT-3', Prig-R: 5'-ACGCCATCCCCATCCCTTA-3', Prig-P: 5'-GACGGCATCCGATATGAAACAAAGG-3', Pseu-F: 5'-TGGGATCACCACCTCTACTT-3', Pseu-R: 5'-TTTCGGTGTACGTCAGA-3', Pseu-P: 5'-AGTACGACAGCTCCGACCTGAAGCCG-3',	16s rRNA	59.0 60.9	42.8 55.0	21 20	This study
* <i>B. vulgatus</i>	Bvul-F: 5'-CGGGCTTAATTCCAGATGA-3', Bvul-R: 5'-CATGCAGCACCTCACAGAT-3', Bvul-P: 5'-GGTGAAGGCCGTAAGCCGCAAGG-3', Bwads-F: 5'-GGCTGGAAACGGTGCCTAA-3', Bwads-R: 5'-GGACTCATCCTTAAGGGATAGC-3', Bwads-P: 5'-CGAATACGCTCCGATTATCATTTGGGG-3', Prev-F: 5'-CCAGCCAAGTAGCGTGCA-3', Prev-R: 5'-TGGACCTCCGTATTACCGC-3', Prev-P: 5'-CAAATCTGATGCCGTCATCGAAGACTATGC-3', Prig-F: 5'-GGCCTAATACCCGATGTGTT-3', Prig-R: 5'-ACGCCATCCCCATCCCTTA-3', Prig-P: 5'-GACGGCATCCGATATGAAACAAAGG-3', Pseu-F: 5'-TGGGATCACCACCTCTACTT-3', Pseu-R: 5'-TTTCGGTGTACGTCAGA-3', Pseu-P: 5'-AGTACGACAGCTCCGACCTGAAGCCG-3',	16s rRNA	58.9 59.0	47.6 52.6	21 19	This study
* <i>B. wadsworthia</i>	Bwads-F: 5'-GGCTGGAAACGGTGCCTAA-3', Bwads-R: 5'-GGACTCATCCTTAAGGGATAGC-3', Bwads-P: 5'-CGAATACGCTCCGATTATCATTTGGGG-3', Prev-F: 5'-CCAGCCAAGTAGCGTGCA-3', Prev-R: 5'-TGGACCTCCGTATTACCGC-3', Prev-P: 5'-CAAATCTGATGCCGTCATCGAAGACTATGC-3', Prig-F: 5'-GGCCTAATACCCGATGTGTT-3', Prig-R: 5'-ACGCCATCCCCATCCCTTA-3', Prig-P: 5'-GACGGCATCCGATATGAAACAAAGG-3', Pseu-F: 5'-TGGGATCACCACCTCTACTT-3', Pseu-R: 5'-TTTCGGTGTACGTCAGA-3', Pseu-P: 5'-AGTACGACAGCTCCGACCTGAAGCCG-3',	16s rRNA	61.0 60.8	57.8 60.8	23 23	This study
* <i>Prevotella</i> spp.	Bvul-F: 5'-GGCTGGAAACGGTGCCTAA-3', Bvul-R: 5'-GGACTCATCCTTAAGGGATAGC-3', Bvul-P: 5'-CGAATACGCTCCGATTATCATTTGGGG-3', Prev-F: 5'-CCAGCCAAGTAGCGTGCA-3', Prev-R: 5'-TGGACCTCCGTATTACCGC-3', Prev-P: 5'-CAAATCTGATGCCGTCATCGAAGACTATGC-3', Prig-F: 5'-GGCCTAATACCCGATGTGTT-3', Prig-R: 5'-ACGCCATCCCCATCCCTTA-3', Prig-P: 5'-GACGGCATCCGATATGAAACAAAGG-3', Pseu-F: 5'-TGGGATCACCACCTCTACTT-3', Pseu-R: 5'-TTTCGGTGTACGTCAGA-3', Pseu-P: 5'-AGTACGACAGCTCCGACCTGAAGCCG-3',	16s rRNA	61.0 70.8	57.8 60.8	19 23	This study
* <i>P. nigrescens</i>	Bvul-F: 5'-GGCTGGAAACGGTGCCTAA-3', Bvul-R: 5'-GGACTCATCCTTAAGGGATAGC-3', Bvul-P: 5'-CGAATACGCTCCGATTATCATTTGGGG-3', Prev-F: 5'-CCAGCCAAGTAGCGTGCA-3', Prev-R: 5'-TGGACCTCCGTATTACCGC-3', Prev-P: 5'-CAAATCTGATGCCGTCATCGAAGACTATGC-3', Prig-F: 5'-GGCCTAATACCCGATGTGTT-3', Prig-R: 5'-ACGCCATCCCCATCCCTTA-3', Prig-P: 5'-GACGGCATCCGATATGAAACAAAGG-3', Pseu-F: 5'-TGGGATCACCACCTCTACTT-3', Pseu-R: 5'-TTTCGGTGTACGTCAGA-3', Pseu-P: 5'-AGTACGACAGCTCCGACCTGAAGCCG-3',	16s rRNA	61.1 70.0	58.6 46.1	18 26	[28]
* <i>P. aeruginosa</i>	Bvul-F: 5'-GGCTGGAAACGGTGCCTAA-3', Bvul-R: 5'-GGACTCATCCTTAAGGGATAGC-3', Bvul-P: 5'-CGAATACGCTCCGATTATCATTTGGGG-3', Prev-F: 5'-CCAGCCAAGTAGCGTGCA-3', Prev-R: 5'-TGGACCTCCGTATTACCGC-3', Prev-P: 5'-CAAATCTGATGCCGTCATCGAAGACTATGC-3', Prig-F: 5'-GGCCTAATACCCGATGTGTT-3', Prig-R: 5'-ACGCCATCCCCATCCCTTA-3', Prig-P: 5'-GACGGCATCCGATATGAAACAAAGG-3', Pseu-F: 5'-TGGGATCACCACCTCTACTT-3', Pseu-R: 5'-TTTCGGTGTACGTCAGA-3', Pseu-P: 5'-AGTACGACAGCTCCGACCTGAAGCCG-3',	16s rRNA	61.5 71.2	58.9 59.0	21 19	This study

Target organism	Sequence of primer or probe genes (5' end labeled with FAM-6 and 3' end t	T _m	GC%	Length	Refere nce
<i>*P. disiens</i>	Pdis-F: 5'-GCCGGTACAGAAATGTTGGTT-3' Pdis-R: 5'-GGTCGTGAGGTCGGTT-3' Pdis-P: 5'-AATGTAAATCTAATCCTTAAGCCGGTCCAGGTT-3' Pbiv-F: 5'-GGTGGTACAGATAAGTTGGTCGTG-3' Pbiv-R: 5'-TTGCGAGCCCCAGTCCGA-3' Pbiv-P: 5'-GCAAATACGATCTAACCTTAAAACCATTCTCAG-3' Pmel-F: 5'-GTGGGATAACCTGCCGAAAG-3' Pmel-R: 5'-CCCATACCGATAAAATCTTTA-3' Pmel-P: 5'-GCATAGTCTCGATGACGGCATCAGATTG-3' Pmac-F: 5'-TTGAAATTAGGGACTATGTATG-3' Pmac-R: 5'-AGCTGACGACAACCATGCA-3' Pmac-P: 5'-TACATATCCTGTACAAGGCCGCTAACGTAGG-3'	16S rRNA	57.4 57.6	50.0 61.1	20 18
<i>*P. bivia</i>		16S rRNA	71.5 58.8	38.2 52.1	34 23
<i>P. melaninogenica</i>		16S rRNA	59.9	61.1	18
<i>*Porphyromonas macacae</i>		16S rRNA	69.5	35.3	34
<i>Fusobacterium</i> spp.	Fuso-F: 5'-AAGCGCGCTAGGTGGTTATGT-3' Fuso-R: 5'-TGTAGTCCGGCTTACCTCTCCAG-3' Fuso-P: 5'-CAACGCAATAACAGAGTTGAGGCCCTGCATT-3' Fmag-F: 5'-AGGGTACGCAGGGGTTAAT-3' Fmag-R: 5'-TCAGTTCCAATGCCTTACGG-3' Fmag-P: 5'-AGTCGAATGTTAAAGATCGGGGCTCAAC-3' Pmic-F: 5'-GCCGTGGAAACCGACGAATTA-3' Pmic-R: 5'-CACCGATAAAATCTTGACCCCTAT-3' Pmic-P: 5'-TACCGCATGAGACCACAGAAATCGCA-3' Pana-F: 5'-GGCGTAAGGGTGCCTAGGT-3' Pana-R: 5'-TCGGAGGCTAACTACGGTTGA-3' Pana-P: 5'-TCCTCAAGTCGGTGGTTAAAGGCTACGG-3' Aodo-F: 5'-ATGTGGTGGCATTTAGTTGGTC-3' Aodo-R: 5'-GACTTAACCCAAACATCTCACGAC-3' Aodo-P: 5'-GGTGGTGCATGGTGTGAGCTCG-3'	16S rRNA	65.8 60.6	50.0 52.1	22 23
<i>*Finegoldia magna</i>		16S rRNA	73.1	48.2	29
<i>*Micromonas micros</i>	Fmag-F: 5'-AGGGTACGCAGGGTTAAT-3' Fmag-R: 5'-TCAGTTCCAATGCCTTACGG-3' Fmag-P: 5'-AGTCGAATGTTAAAGATCGGGGCTCAAC-3' Pmic-F: 5'-GCCGTGGAAACCGACGAATTA-3' Pmic-R: 5'-CACCGATAAAATCTTGACCCCTAT-3' Pmic-P: 5'-TACCGCATGAGACCACAGAAATCGCA-3' Pana-F: 5'-GGCGTAAGGGTGCCTAGGT-3' Pana-R: 5'-TCGGAGGCTAACTACGGTTGA-3' Pana-P: 5'-TCCTCAAGTCGGTGGTTAAAGGCTACGG-3' Aodo-F: 5'-ATGTGGTGGCATTTAGTTGGTC-3' Aodo-R: 5'-GACTTAACCCAAACATCTCACGAC-3' Aodo-P: 5'-GGTGGTGCATGGTGTGAGCTCG-3'	16S rRNA	62.8 58.2	52.3 42.8	21 21
<i>*P. anaerobius</i>		16S rRNA	70.0	46.4	28
<i>*Actinomyces odontolyticus (& A. lingnae)</i>		16S rRNA	61.0	50.0	20
		16S rRNA	61.7	60.0	20
		Pana-R: 5'-TCGGAGGCTAACTACGGTTGA-3' Pana-P: 5'-TCCTCAAGTCGGTGGTTAAAGGCTACGG-3' Aodo-F: 5'-ATGTGGTGGCATTTAGTTGGTC-3' Aodo-R: 5'-GACTTAACCCAAACATCTCACGAC-3' Aodo-P: 5'-GGTGGTGCATGGTGTGAGCTCG-3'	61.6	50.0	24
		Pana-R: 5'-TCGGAGGCTAACTACGGTTGA-3' Pana-P: 5'-TCCTCAAGTCGGTGGTTAAAGGCTACGG-3' Aodo-F: 5'-ATGTGGTGGCATTTAGTTGGTC-3' Aodo-R: 5'-GACTTAACCCAAACATCTCACGAC-3' Aodo-P: 5'-GGTGGTGCATGGTGTGAGCTCG-3'	71.6	48.2	29
		Aodo-F: 5'-ATGTGGTGGCATTTAGTTGGTC-3' Aodo-R: 5'-GACTTAACCCAAACATCTCACGAC-3' Aodo-P: 5'-GGTGGTGCATGGTGTGAGCTCG-3'	59.0	45.4	22
		Aodo-F: 5'-ATGTGGTGGCATTTAGTTGGTC-3' Aodo-R: 5'-GACTTAACCCAAACATCTCACGAC-3' Aodo-P: 5'-GGTGGTGCATGGTGTGAGCTCG-3'	58.1	47.8	23
		Aodo-F: 5'-ATGTGGTGGCATTTAGTTGGTC-3' Aodo-R: 5'-GACTTAACCCAAACATCTCACGAC-3' Aodo-P: 5'-GGTGGTGCATGGTGTGAGCTCG-3'	75.4	61.5	26

<i>Clostridium perfringens</i>	CPerF165F: 5'-CGCATAACGTTGAAAGATGG-3' CPerf269R: 5'-CCTTGGTAGGCCGTTACCC-3' Cperf-P: 5'-TCATCATCAACCAAAGGAGCAATCC-3' Staph-F: 5'-CAAC(T/A)CCAGAACGTGATT(C)CTG-3' Staph-R: 5'-CAGTACCCACGACCAGTGTGAG-3' Staph-P: 5'-AAACCATTCATGATGCCAAGTTGAGGACG-3' Saur-F: 5'-AGCTCAGCAAATGCATCACAA-3' Saur-R: 5'-TTAGTTGAAGTTGCACTATATACTGTTGGAT-3' Saur-P: 5'-CAGATA(C/T)GGCGTAATAGAAAGTGGTC-3' Sepi-F: 5'-TACACACCGCCCGTCACA-3' Sepi-R: 5'-CTTCGACGGGCTAGCTCCAAT-3'	16S rRNA	56.8 59.4 67.5 58.6 59.6 72.0 59.2 62.6 67.5 59.0 64.7 70.5 58.6 53.9 69.0 51.4 51.8 61.67 60.7 61.9 72.1 60.0 60.0 71.2 64.0 64.4 74.9	20 19 26 21 23 28 21 21 31 32 18 22 23 20 20 30 23 20 33 25 24 34 21 25 29 28 24 33.3 42.4	[29] present study present study [30]
<i>*Staphylococcus</i> spp.					
<i>* S. aureus</i>					
<i>S. epidermidis</i>					
<i>MRSA</i>					
<i>PVL</i>					
<i>*Group A</i>					
<i>Streptococcus</i>					
<i>*Group B</i>					
<i>Streptococcus</i>					
<i>*Anginosus group streptococci</i>					

* Primers and probes were designed in present study.

(4). Specificity evaluation for TaqMan PCR assay:

The specificities of primer/probe sets designed in this study were predicted by comparison to the aligned SSU_rRNA database of the RDP using the CHECK_PROBE utility [21] and to all available sequences by using the BLAST database search program (www.ncbi.nlm.nih.gov/BLAST) [22], and further confirmed by testing against a panel of phylogenetically related bacterial strains. The specificities of the rest of the primer/probe sets have previously been demonstrated [28-32].

Gram-negative anaerobes:

The specificities of the primer and probe sets for *Bacteroides fragilis*, *B. thetaiotaomicron*, *B. stercoris*, *B. vulgatus*, *Bilophila wadsworthia*, *Prevotella nigrescens*, *P. disiens*, *P. bivia* and *Porphyromonas macacae* were tested against a panel of genomic DNA preparations (Table 2 & 3) from type strains and well-characterized clinical isolates that are phylogenetically related to these species. The primer/probe sets detected only corresponding strain(s), except for that of *B. ovatus* which showed a significant increase in fluorescence indicating cross-reactivity with *B. thetaiotaomicron* species-specific primer and probe. No signal was detected for any other non-corresponding strains.

Table 2. Bacterial strains tested by TaqMan assay

Species	Strains	No. of Strains	Bthe-F/R/P	Bfrag-F/R/P	Bster-F/R/P	Bvul-F/R/P	Pnig-F/R/P	Pdis-F/R/P	Pbiv-F/R/P
<i>Bacteroides thetaiotaomicron</i>	ATCC 29148 ^T	1	+	-	-	-	NT	NT	NT
<i>Bacteroides thetaiotaomicron</i>	Clinical isolates	6	+	-	-	-	NT	NT	NT
<i>Bacteroides ovatus</i>	ATCC 8483 ^T	1	+	-	-	-	NT	NT	NT
<i>Bacteroides ovatus</i>	Clinical isolates	6	+	-	-	-	NT	NT	NT
<i>Bacteroides fragilis</i>	ATCC 25285 ^T	1	-	+	-	-	NT	NT	NT
<i>Bacteroides fragilis</i>	Clinical isolates	6	-	+	-	-	NT	NT	NT
<i>Bacteroides stercoris</i>	ATCC 43183 ^T	1	-	-	+	-	NT	NT	NT
<i>Bacteroides stercoris</i>	Clinical isolates	6	-	-	+	-	NT	NT	NT
<i>Bacteroides vulgatus</i>	ATCC 8482 ^T	1	-	-	-	+	NT	NT	NT
<i>Bacteroides vulgatus</i>	Clinical isolates	6	-	-	-	+	NT	NT	NT
<i>Bacteroides distasonis</i>	ATCC 8503 ^T	1	-	-	-	-	NT	NT	NT
<i>Bacteroides merdae</i>	ATCC 43184 ^T	1	-	-	-	-	NT	NT	NT
<i>Bacteroides caccae</i>	ATCC 43185 ^T	1	-	-	-	-	NT	NT	NT
<i>Bacteroides uniformis</i>	ATCC 8492 ^T	1	-	-	-	-	NT	NT	NT
<i>Bacteroides eggerthii</i>	ATCC 27754 ^T	1	-	-	-	-	NT	NT	NT
<i>Bacteroides goldsteinii</i>	ATCC BAA- 1180 ^T	1	-	-	-	-	NT	NT	NT

Table 2 (Cont.)

<i>Bacteroides nordii</i>	ATCC BAA- 998 ^T	1	-	-	-	-	-	NT	NT	NT
<i>Bacteroides salyersiae</i>	ATCC BAA-997 ^T	1	-	-	-	-	-	NT	NT	NT
<i>Prevotella nigrescens</i>	ATCC 33563 ^T	1	NT	NT	NT	NT	+	-	-	-
<i>Prevotella nigrescens</i>	Clinical isolates	5	NT	NT	NT	NT	+	-	-	-
<i>Prevotella disiens</i>	ATCC 29426 ^T	1	NT	NT	NT	NT	-	+	-	-
<i>Prevotella disiens</i>	Clinical isolates	5	NT	NT	NT	NT	-	+	-	-
<i>Prevotella bivia</i>	ATCC 29303 ^T	1	NT	NT	NT	NT	-	-	-	+
<i>Prevotella bivia</i>	Clinical isolates	3	NT	NT	NT	NT	-	-	-	+
<i>Prevotella melaninogenica</i>	ATCC 25845 ^T	1	NT	NT	NT	NT	-	-	-	-
<i>Prevotella buccae</i>	ATCC 33574 ^T	1	NT	NT	NT	NT	-	-	-	-
<i>Prevotella buccalis</i>	ATCC 35310 ^T	1	NT	NT	NT	NT	-	-	-	-
<i>Prevotella corporis</i>	ATCC 33547 ^T	1	NT	NT	NT	NT	-	-	-	-
<i>Prevotella denticola</i>	ATCC 33185	1	NT	NT	NT	NT	-	-	-	-
<i>Prevotella intermedia</i>	ATCC 25611 ^T	1	NT	NT	NT	NT	-	-	-	-
<i>Prevotella loescheii</i>	ATCC 15930 ^T	1	NT	NT	NT	NT	-	-	-	-
<i>Prevotella oralis</i>	ATCC 33269 ^T	1	NT	NT	NT	NT	-	-	-	-
<i>Prevotella oulorum</i>	ATCC 43324 ^T	1	NT	NT	NT	NT	-	-	-	-
<i>Prevotella veroralis</i>	ATCC 33779 ^T	1	NT	NT	NT	NT	-	-	-	-
<i>Prevotella zoogloeformans</i>	ATCC 33285 ^T	1	NT	NT	NT	NT	-	-	-	-
<i>Porphyromonas gingivalis</i>	ATCC 33277 ^T	1	NT	NT	NT	NT	-	-	-	-
<i>Porphyromonas endodontalis</i>	ATCC 35406 ^T	1	NT	NT	NT	NT	-	-	-	-

Table 3. Bacterial strains tested by TaqMan assay

Species	Strains	No. of Strains	Pmac-F/R/P	Bwads-F/R/P
<i>Porphyromonas macacae</i>	ATCC 33141 ^T	1	+	NT
<i>Porphyromonas asaccharolytica</i>	ATCC 25260 ^T	1	-	NT
<i>Porphyromonas endodontalis</i>	ATCC 35406 ^T	1	-	NT
<i>Porphyromonas levii</i>	ATCC 29147 ^T	1	-	NT
<i>Porphyromonas gingivalis</i>	ATCC 33277 ^T	1	-	NT

<i>Porphyromonas gingivalis</i>	ATCC 49417	1	-	NT
<i>Porphyromonasансulci</i>	NCTC 12858 ^T	1	-	NT
<i>Porphyromonas cangingivalis</i>	NCTC 12856 ^T	1	-	NT
<i>Porphyromonas canoris</i>	NCTC 12835 ^T	1	-	NT
<i>Porphyromonas somerae</i>	ATCC BAA-1230 ^T	1	-	NT
<i>Porphyromonas uenonis</i>	ATCC BAA-906 ^T	1	-	NT
<i>Porphyromonas gulae</i>	ATCC 51700 ^T	1	-	NT
<i>Tannerella forsythensis</i>	ATCC 43037	1	NT	-
<i>Bilophila wadsworthia</i>	ATCC 49260 ^T	1	NT	+
<i>Bilophila wadsworthia</i>	ATCC 51581	1	NT	+
<i>Bilophila wadsworthia</i>	Clinical isolates	8	NT	+
<i>Desulfovibrio piger</i>	ATCC 29098 ^T	1	NT	-
<i>Desulfovibrio piger</i>	Clinical isolates	4	NT	-
<i>Desulfovibrio desulfuricans</i>	ATCC 7757	1	NT	-
<i>Desulfovibrio desulfuricans</i>	Clinical isolates	4	NT	-
<i>Sutterella wadsworthensis</i>	ATCC 51579 ^T	1	NT	-
<i>Desulfovibrio fairfieldensis</i>	Clinical isolates	2	NT	-
<i>Desulfovibrio vulgaris</i>	Clinical isolates	2	NT	-

NT: not tested

Gram-positive anaerobes:

The specificities of the primer and probe sets for *F. magna*, *M. micros* and *P. anaerobius* were tested by running PCR with DNA samples from 14 type strains and 17 well-characterized clinical isolates that are phylogenetically related to GPAC species; this resulted in amplification only with the DNA from the corresponding GPAC reference strain(s). None of the non-corresponding organisms tested by the PCR showed an increase in fluorescence to indicate cross-activity with the species-specific primer/probe sets (See table 4).

Comparison of the sequences of primer/probe set for *Actinomyces odontolyticus* to the aligned SSU_rRNA database of the RDP using the CHECK_PROBE utility predicted it cross-reacts with *A. lingnae*. Further tests will be done with this set of oligonucleotides in the future.

Table 4: Bacterial strains tested by TaqMan assay

Species	Strains	No.	Fmag-F/R/P	Pmic-F/R/P	Pana-F/R/P
<i>Finegoldia magna</i>	CCUG 17636 ^T	1	+	-	-
<i>Finegoldia magna</i>	Clinical isolates	6	+	-	-
<i>Micromonas micros</i>	ATCC 33270 ^T	1	-	+	-
<i>Micromonas micros</i>	Clinical isolates	6	-	+	-
<i>Peptostreptococcus anaerobius</i>	CCUG 7835 ^T	1	-	-	+
<i>Peptostreptococcus anaerobius</i>	Clinical isolates	5	-	-	+
<i>Anaerococcus hydrogenalis</i>	ATCC 49630 ^T	1	-	-	-
<i>Anaerococcus lactolyticus</i>	CCUG 31351 ^T	1	-	-	-
<i>Anaerococcus octavius</i>	CCUG 38493 ^T	1	-	-	-
<i>Anaerococcus prevotii</i>	CCUG 41932 ^T	1	-	-	-
<i>Anaerococcus tetradius</i>	CCUG 46590 ^T	1	-	-	-
<i>Anaerococcus vaginalis</i>	CCUG 31349 ^T	1	-	-	-
<i>Peptoniphilus. asaccharolyticus</i>	CCUG 9988 ^T	1	-	-	-
<i>Peptoniphilus harei</i>	CCUG 38491 ^T	1	-	-	-
<i>Peptoniphilus indolicus</i>	CCUG 17639 ^T	1	-	-	-
<i>Peptoniphilus ivorii</i>	CCUG 38492 ^T	1	-	-	-
<i>Peptoniphilus lacrimalis</i>	CCUG 31350 ^T	1	-	-	-

Gram-positive aerobes:

Anginosus group and Group A and B streptococci:

As the non-anginosus group streptococci, the 14 reference strains representing 12 different species (**Table 5**) were used. As the anginosus streptococci, the type strain (CCUG 27298^T) and three isolates of *S. anginosus*, the type strain (ATCC 27283^T), one reference strain (CCUG 24889) and one isolate of *S. constellatus* subsp. *constellatus*, and the type strain (CCUG 32759^T) and two isolates of *S. intermedius* were used. The clinical strains of the anginosus group were recovered from our facility. The clinical isolates were identified by routine biochemical methods, which included evaluation by use of the Vitek and API systems. None of the 14 non-anginosus organisms tested by the PCR showed an increase in fluorescence to indicate cross-reactivity with the primer/probe sets (Table 5). Conversely, all the anginosus streptococci isolates showed an exponential increase in fluorescence. Similarly, the primer/probe sets for Group A and Group B streptococci were tested against a battery of streptococci (Table 5), and fluorescent signal were only detected from corresponding strains.

Staphylococcus spp. & *S. aureus*:

An analysis of the bacterial battery demonstrated the specificities of our primers and probes. The broad-range primer/probe set designed for the *Staphylococcus* genus generated positive curves for all of the staphylococci tested (Fig.2.). The *S. aureus*-specific assay detected only the *S. aureus* isolates in the battery (Table 5).

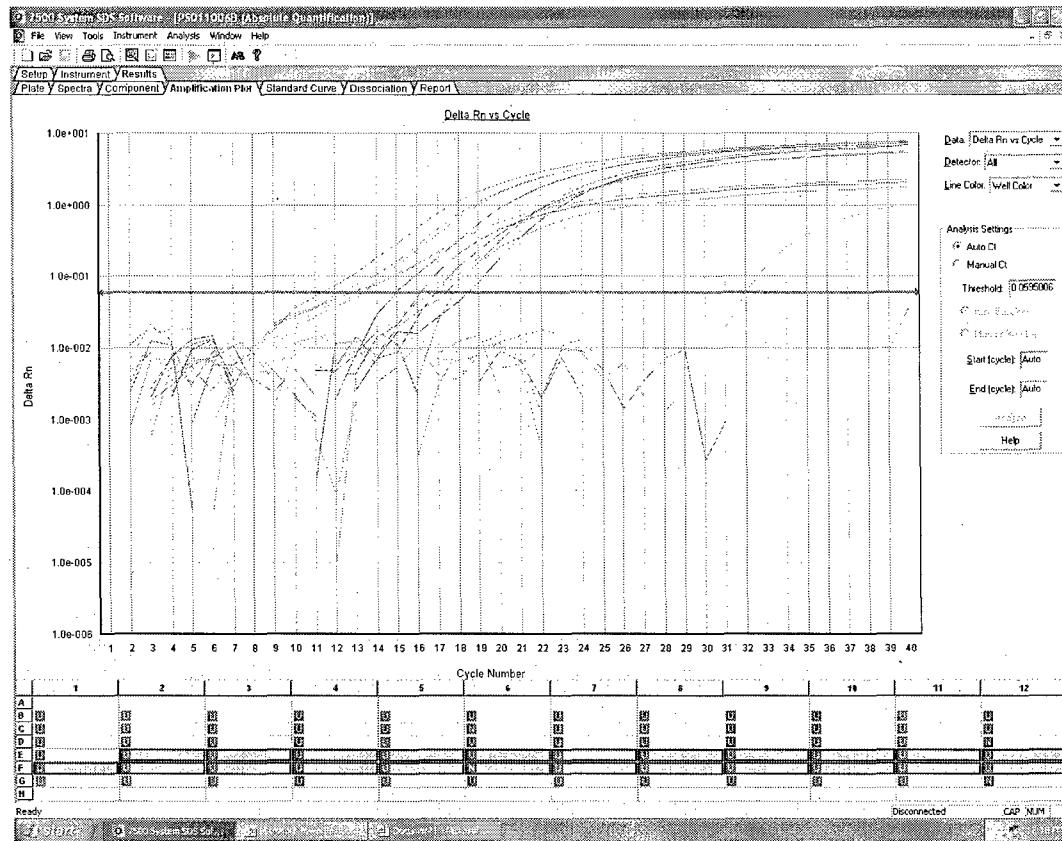


Fig. 2. Example showing the FAM fluorescence detection of *Staphylococcus* spp.

Gram-negative aerobe:

The specificity of the primer/probe set devised for *Pseudomonas aeruginosa* was predicted by comparison to the aligned SSU_rRNA database of the RDP using the CHECK_PROBE utility and was compared to all available 16S rDNA sequences by using the BLAST database search program. Theoretical cross-specificity analysis indicated that the detection system would be specific for *Pseudomonas aeruginosa* at the species level. The primer/probe set was tested against two *Pseudomonas aeruginosa* strains (type strain ATCC 10145 and reference strain ATCC 27853) and the other two *Pseudomonas* strains including the type strain of *Pseudomonas fluorescens* (ATCC 13525^T) and the type strain of *Pseudomonas putida* (ATCC 12633^T). The fluorescent signal was only detected from *Pseudomonas aeruginosa* strains. Specificity of the primer/probe set needs to be further confirmed by testing more phylogenetically related strains in the future.

Table 5. Bacterial strains tested by TaqMan assay

Species	Strains	No.	Staphy-F/R/P	Saur-F/R/P	GAS-F/R/P	GBS-F/R/P	AGS-F/R/P
<i>Staphylococcus</i> spp.							
<i>S. aureus</i>	ATCC 29213	1	+	+	NT	NT	NT
<i>S. aureus</i>	ATCC 25923	1	+	+	NT	NT	NT
<i>S. aureus</i>	Clinical isolates	4	+	+	NT	NT	NT
<i>S. epidermidis</i>	ATCC 12228	1	+	-	NT	NT	NT
<i>S. epidermidis</i>	ATCC 49134	1	+	-	NT	NT	NT
<i>S. epidermidis</i>	Clinical isolates	2	+	-	NT	NT	NT
<i>S. haemolyticus</i>	ATCC 29970 ^T	1	+	-	NT	NT	NT
<i>S. haemolyticus</i>	WAL 1020	1	+	-	NT	NT	NT
<i>S. hominis</i>	WAL 937	1	+	-	NT	NT	NT
<i>S. lugdunensis</i>	ATCC 49576	1	+	-	NT	NT	NT
<i>S. saprophyticus</i>	ATCC 15305 ^T	1	+	-	NT	NT	NT
<i>S. simulans</i>	ATCC 27851	1	+	-	NT	NT	NT
<i>S. warneri</i>	ATCC 49454	1	+	-	NT	NT	NT
<i>S. warneri</i>	WALA 939	1	+	-	NT	NT	NT
<i>S. xylosus</i>	ATCC 29971 ^T	1	+	-	NT	NT	NT
CoNS <i>Staphylococcus</i>	Clinical isolates	7	+	-	NT	NT	NT
<i>Streptococcus</i> spp.							
<i>S. bovis</i>	ATCC 35034	1	-	NT	-	-	-
<i>S. dysgalactiae</i> subsp. <i>equisimilis</i>	ATCC 35666	1	NT	NT	-	-	-
<i>S. mitis</i>	ATCC 49456 ^T	1	-	NT	-	-	-
<i>S. mutans</i>	ATCC 25175 ^T	1	-	NT	-	-	-
<i>S. oralis</i>	ATCC 9811	1	NT	NT	-	-	-
<i>S. salivarius</i>	ATCC 7073 ^T	1	-	NT	-	-	-
<i>S. sanguinis</i>	ATCC 10556 ^T	1	-	NT	-	-	-
<i>S. vestibularis</i>	ATCC 49124 ^T	1	-	NT	-	-	-
<i>S. anginosus</i>	CCUG 27298 ^T	1	NT	NT	NT	-	+
<i>S. anginosus</i>	Clinical isolates	3	NT	NT	NT	NT	+
<i>S. agalactiae</i>	ATCC 12386	1	NT	NT	-	+	-
<i>S. agalactiae</i>	ATCC 13813 ^T	1	NT	NT	-	+	-

<i>S. agalactiae</i>	Clinical isolates	2	NT	NT	NT	+	NT
<i>S. iniae</i>	ATCC 29178 ^T	1	NT	NT	NT	-	-
<i>S. constellatus</i> subsp. <i>constellatus</i>	ATCC 27823 ^T	1	NT	NT	-	-	+
<i>S. constellatus</i>	CCUG 24889	1	NT	NT	NT	-	+
<i>S. constellatus</i>	WALA 154	1	NT	NT	NT	NT	+
<i>S. intermedius</i>	CCUG 32759	1	NT	NT	NT	NT	+
<i>S. intermedius</i>	Clinical isolates	2	NT	NT	NT	NT	+
<i>S. pneumoniae</i>	ATCC 35088	1	NT	NT	-	-	-
<i>S. pneumoniae</i>	ATCC 49615	1	NT	NT	NT	-	NT
<i>S. pneumoniae</i>	ATCC 6305	1	NT	NT	NT	-	NT
<i>S. pyogenes</i>	ATCC 12384	1	NT	NT	+	-	-
<i>S. pyogenes</i>	ATCC 19615	1	NT	NT	+	-	-
<i>S. pyogenes</i>	Clinical isolates	2	NT	NT	+	-	NT

NT: not tested

KEY RESEARCH ACCOMPLISHMENTS

Rapid identification of pathogens involved in wound infections is critical for successfully controlling, managing and eradicating this type of infection. Rapid field-based tests to detect the pathogens involved in the infections are not available. The most significant accomplishment during the last year was development of a rapid real-time TaqMan PCR assay for most of the pathogens encountered in wound infections. This rapid and specific assay provides a new diagnostic tool that conceivably will redefine wound infection management and control strategies for this important problem in both military and civilian life. Evaluation of the sensitivity of the assay is underway.

REPORTABLE OUTCOMES

When the entire set of primers and probes has been completed, this information plus the methodology for real-time PCR using these will be published.

CONCLUSION

We have developed a real-time PCR based high throughput detection method for certain important wound pathogens.

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